Determination of Nicotine and its Metabolites in Urine by HPLC after DETBA Derivatization

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Introduction

Nicotine is rapidly metabolized and a large number of phase 1 and phase 2 metabolites are excreted in urine (Gorrod and Schepers, 1989). The quantitative determination of these metabolites in urine collected over 24 h is a generally accepted method for biomonitoring nicotine uptake. Due to the high interindividual variance in nicotine metabolism, the quality of biomonitoring would improve if nicotine and all of its metabolites were determined.

An extensive number of methods for the determination of nicotine and its metabolites have been published. These include numerous HPLC-, GC-, and immunological methods (for review see Davies and Garvan, 1990), most of them can only determine a limited number of metabolites. It is not possible with any of the published methods to determine all major nicotine metabolites in a single analytical process, e.g., the analysis of the glucuronides is generally performed by an indirect method, i.e., by analysis of the aglycon before and after deconjugation.

In 1993 we published an HPLC method for the determination of nicotine and a large number of its metabolites after derivatization with 1,3-diethyl-2-thiobarbituric acid (DETBA) (Rustemeier et al., 1993). With this method, the determination of cotinine and nicotine-N-oxide requires a separate chromatographic run at elevated temperature. Each chromatographic run takes 37 min, which means that complete analysis of one urine sample including the analysis of the glucuronides takes about 2 h (3 runs).

To improve the method and shorten the time required, we modified the derivatization conditions, solvent composition, pH, flow, and detection wavelengths and automated the method.

Methods

Reference Compounds

Metabolite: Abbreviation	Name	Supplier
PHOBAC	4-(3-pyridyl)-4-hydroxybutyric acid	TRC, Toronto, Canada
PAAC	3-pyridylacetic acid	Sigma-Aldrich, Taufkirchen, Germany
6-HNICOT	6-hydroxynicotine	Lyonnais, Hamburg, Germany
3'HNICOT	3'-hydroxycotinine (trans + cis)	TRC, Toronto, Canada (synth.)
NICOT	nicotinone	TRC, Toronto, Canada
COT	cotinine	Sigma-Aldrich, Taufkirchen, Germany
NNO	nicotine-N'-oxide	TRC, Toronto, Canada
NMIC	nornicotine	Sigma-Aldrich, Taufkirchen, Germany
NIC	nicotine	Sigma-Aldrich, Taufkirchen, Germany
PCBAC	4-(3-pyridyl)-4-oxobutyric acid	TRC, Toronto, Canada
CNO	cotinine-N-oxide	TRC, Toronto, Canada
IS	enantiomeric mixture of 3-ethylnicotine	Merk, Darmstadt, Germany

Preparation

urine from adult (over 21) smokers

Enzymatic Deconjugation Treatment

- 500 µl urine sample
  + 100 µl β-glucuronidase (from Helix pomatia, Type H-1); (80,000 units/ml, in 0.25 mol/l acetate buffer pH 4.7)
- thoroughly mixed
- incubated for ~20 h at 37°C
- centrifuged

Derivatization Procedure

(derivatization and incubation performed at 4°C)

- 100 µl sample (centrifuged urine or β-glucuronidase-treated urine)
  + 5 µl internal standard (IS) (IS: N-ethylnornicotine, 10 mg/l H_2O)
  + 10 µl ammonium acetate (0.35 mol/l H_2O)
  + 5 µl potassium cyanide (0.8 mol/l H_2O)
  + 5 µl chloramine T (0.45 mol/l H_2O)
  + 5 µl 1,3-diethyl-2-thiobarbituric acid (DETBA) (1.0 mol/l acetone)
- thoroughly mixed
- incubated for exactly 10 min, then injected into HPLC

Chromatography

System: HPLC HP1090L equipped with a ternary solvent delivery system, an additional 6-port rotary valve, and an HP1100 diode array detector; controlled by HP ChemStation

Autosampler: HTS PAL equipped with Peltier-cooled sample rack

Precolumn: Security Guard, Max-RP (4 mm x 3 mm)

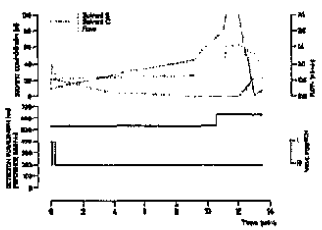
Column: Synergi Max-RP, 80 A, (150 mm x 2.0 mm; 4 µm)

Oven temp.: 38°C

Injection vol.: up to 100 µl

Solvents: A: aqueous solution of 1-pentanesulfonic acid (9.5 g/l), 1-heptanesulfonic acid (2.7 g/l), 1-octanesulfonic acid (0.8 g/l), and ammonium acetate (0.15 g/l) adjusted with acetic acid (10 %) to pH 4.72 (conductivity: 4.3 ms/cm)
B: ammonium acetate (0.45 g/l methanol)
C: acetonitrile/tetrahydrofuran (96/4, v/v)

Solvent, Gradient, Flow, Valve Setting, and Detection Wavelength:



Results and Discussion

The method presented here is fully automated. Derivatization (which can also be performed in a non-automated manner) and chromatography are staggered in order to perform a chromatographic run every 13.5 min. The method has been optimized for the determination of nicotine and its metabolites in human urine but we also use it routinely (with some minor modifications) for biomonitoring nicotine uptake in rats exposed to cigarette smoke (Heuzenroeder et al., 1995).

Calibration curves for each individual metabolite are linear over a wide concentration range. The response of the derivatized products varies considerably (linear regression coefficient between 0.08 and 2.7 nmol^{-1}). The lowest response and the highest detection limit was found for PCBAC, which is a minor nicotine metabolite in smokers (~0.3 %) but a major metabolite found in the urine of nicotine-treated rats (10 to 14 %, Schepers et al., 1993).

Due to the short run time, no separation between the *trans*- and *cis*-stereoisomers of 3'HNICOT is achieved (in human urine: *cis*- <1 % of the *trans*-isomer; the abundance of a significantly higher percentage of the *cis*-isomer (which was not seen in any of the samples analyzed) would be indicated by a shoulder at the beginning of the peak of 3'HNICOT). In addition, no analysis of cotinine-N-oxide (CNO) was performed, as this metabolite (in human urine: ~3 %) cannot be derivatized directly with DETBA.

The relative distribution of nicotine metabolites in 24-h urine samples from smokers and the high interindividual variability reported in the literature was confirmed and extended in 2 studies on smokers from the US and Sweden.

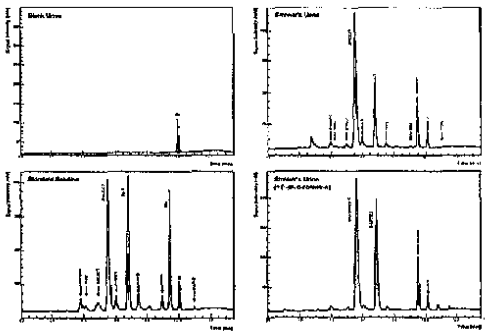
Metabolite	Linear Regression Coefficient,* (r²nmol)	Detection Limit (nmol/l)	Upper Limit of Calculation,* (nmol/l)	Precision (R.S.D. %) Intra-Assay *	Inter-Day *
PHOBAC	0.08	7.5	37	4.2	13.1
PAAC	0.54	10.4	8	5.5	5.0
6'HNICOT	0.48	10.4	37	4.9	9.0
3'HNICOT	2.42	1.5	67	4.9	6.2
NICOT	1.47	7.3	11	2.5	3.8
COT	2.66	3.1	72	3.0	4.8
NNO	0.57	3.2	24	3.3	2.8
NMIC	0.99	2.8	15	4.1	2.3
NIC	2.66	2.4	43	4.6	6.5
PCBAC	0.08	37.8	7.4	9.7	10.4
CNO †	-	-	-	-	-

Linear Regression Coefficient, Detection Limit, and Method Precision

(*) calculated from consumption of radioactive standards (n = 10 x 4 concentrations) (calculated as 6)
Correlation coefficient, r² ≥ 0.99
(†) highest level tested
(‡) n = 10
(§) n = 6 or 8
(¶) CNO can be determined indirectly after reduction with TiCl$_3$ to COT, this would require an additional chromatographic run

Conclusion

The DETBA assay described here can be used to determine nicotine and 12 of its metabolites (including three phase 2 metabolites) in human urine without the need for any clean-up procedure, e.g., extraction. In total up to 98 % of the known metabolites excreted in human urine can be determined. The assay consists of two chromatographic runs, takes less than 30 min to perform, and requires only very small amounts of urine (<1 ml). Compared with published methods, the DETBA assay provides the most complete profile of nicotine metabolites in humans and can also be applied to the analysis of urine samples from laboratory animals.



HPLC Chromatograms of Standard Solution and Urine Samples after DETBA Derivatization

Metabolite	Relative Distribution (%) Literature Studies A	B	C	D	Our Studies E	F	Values given in Literature
NIC	9.2 ± 2.2	8.9 ± 3.8	7.7 ± 4.6	8.9 ± 4.0	13.2 ± 4.0	15.3 ± 5	n.a. ± n.a.
NIC-GLU *	2.6 ± 1.9	4.9 ± 2.5	3.7 ± 2.9	2.3 ± 1.8	2.1 ± 2.0	n.a. ± n.a.	n.a. ± n.a.
COT	11.8 ± 3.4	11.4 ± 2.7	7.9 ± 2.1	12.4 ± 3.1	10.7 ± n.a.	n.a. ± n.a.	12.0 ± n.a.
COT-GLU *	18.5 ± 6.2	12.6 ± 4.7	11.6 ± 4.4	16.5 ± 8.2	11.4 ± n.a.	n.a. ± n.a.	n.a. ± n.a.
3'HNICOT	31.4 ± 9.7	30.6 ± 10.7	29.4 ± 8.8	34.8 ± 11.1	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.
3'HNICOT-GLU *	7.7 ± 3.3	8.7 ± 5.1	16.8 ± 8.1	5.8 ± 6.8	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.
6'HNICOT	-	-	-	-	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.
NNO	-	-	-	-	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.
NMIC	0.1 ± 0.2	0.9 ± 1.3	2.6 ± 1.7	-	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.
CNO	3.6 ± 1.8	3.2 ± 0.8	9.1 ± 3.7	-	-	-	-
6'HNIC	-	0.6 ± 0.1	-	-	-	-	-
NORNIC	1.4 ± 0.4	-	-	-	-	-	-
PCBAC	-	-	-	14.2 ± 11.5	-	-	-
PHOBAC	-	-	-	7.2 ± 7.5	-	-	-
PAAC	-	-	-	-	-	-	-
SUM	88.2	86.8	91.3	88.6	n.a.	n.a.	n.a.

Relative Distribution (M ± SD) of Nicotine and all of its Metabolites Quantified after 24-h Urine Samples of Smokers; Compilation of 6 Different Studies

A: Byrd et al. (1992), 11 smokers, 7 LC/MS/MS methods
B: Bowman et al. (1994), 12 smokers, 6 different chromatographic (GC, HPLC) methods
C: Zimmermann et al. (1997), 81 smokers, 5 GC methods
D: Hecht et al. (1999), 12 smokers, 4 GC/MS, 3 LC/MS/MS methods
E: 24 smokers, 24 urine samples (June 21) from the U.S. (see Table 1)
F: 100 smokers, 24 urine samples (June 21) from Sweden (see Table 2)

* Glucuronide (GLU) quantified as free after base-hydrolysis minus free (without deconjugation)

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INBIFO is a Philip Morris research laboratory
The Annual SRNT Meeting, Savannah, GA, February 2000

PM3006723257

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